Development of EST microsatellite markers for the Tasmanian palaeoendemic conifer *Lagarostrobos franklinii* (Hook. f.) Quinn (Podocarpaceae)

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Abstract

Nuclear Expressed Sequence Tag (EST) microsatellite markers were developed for the Tasmanian palaeoendemic conifer *Lagarostrobos franklinii* (Hook.-f.) Quinn for genetic studies. RNAseq data was mined for EST microsatellites, and primer pairs were synthesised from 70 contigs with 50 producing amplification products. Of these 50, 10 reliably amplified and displayed polymorphism across 8 samples representing the entire species range. The genetic diversity of these 10 loci was then examined in three wild populations (84 samples). The number of alleles varied from two to thirteen per locus with the average number of alleles per population ranging between 3.0 – 4.7. Observed and expected heterozygosity ranged from 0.34 – 0.42 and 0.37 – 0.44, respectively. Marker cross-amplification was tested in the New Zealand sister species *Manoao colensoi* (Hook. f.) Molloy, but no markers amplified reliably, which possibly reflects the age of divergence between these species (~64 million years). These are the first microsatellite markers developed for the monotypic genus *Lagarostrobos*. They will be valuable for assessing the species extant genetic diversity, the impact of past climatic perturbations and human disturbance and the role of clonal propagation in recruitment.

Keywords: : *Lagarostrobos, Podocarpaceae, EST microsatellites, palaeoendemic, next generation sequencing, huon pine, Tasmania.*

Introduction

The Tasmanian conifer *Lagarostrobos franklinii* (Hook. f.) Quinn (*Podocarpaceae*), or Huon pine, is a slow growing, mostly dioecious evergreen tree that is restricted to high rainfall parts of western and southern Tasmania (Gibson, et al. 1991). Whilst widespread, its distribution is usually restricted to lowland riparian areas (Peterson 1990) where it is a dominant component of the cool temperate rainforest flora. The species is notable for being one of the longest lived trees in the world with individual stems reaching ages of 2500 – 3000 years (Buckley 1997) and one clonal stand in western Tasmania thought to exceed 10,000 years of age (Anker, et al. 2017).

As with other fire sensitive Tasmanian conifers (Worth, et al. 2017), the current range of *L. franklinii* appears to be strongly influenced by fire. This is supported by the fact that the species is absent from a large proportion of its potential climatic range (Read and Busby 1990) especially in areas away from the fire protection afforded by rivers. Significant, rare stands of non-riverine *L. franklinii*, including some near the subalpine zone (Gibson, Davies and Brown 1991), are found only in long unburnt fire refugia (Gibson 1988).

Lagarostrobos franklinii is a conifer of both considerable conservation significance and concern. The species is phylogenetically isolated being the sole member of a diverged, basal, Podocarpaceae genus (Biffin 2011) and is an important component of the Tasmanian palaeoendemic flora (Jordan, et al. 2016). The closest extant relative is the New Zealand endemic

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Manoao colensoi (Hook. f.) Molloy, a monotypic genus erected from *Lagarostrobos* (Molloy 1995). European colonisation of Tasmania has had a significant impact on *L. franklinii* via, logging, hydroelectric inundation, and increased fire frequency and severity (Gibson, Davies and Brown 1991). *Lagarostrobos franklinii* was harvested extensively from the early 1800s until the mid-20th century for its decay resistant timber and consequently, few stands remain untouched (Kerr and McDermott 1999). Overall up to 15 % of its total extent has been lost to hydroelectric schemes (Reid, et al. 1999) while a further 7 % has been impacted by fire (DPIPWE 2011) with very little recovery (Buckley 1997). Furthermore, recent increases in the frequency of fires ignited by dry lightning in western Tasmania (Styger, et al. 2018) pose a severe threat to the species.

Despite intense interest in this iconic species, the only genetic studies to date have used markers with low resolution (i.e. isozymes and chloroplast Sanger sequences) (Clark and Carbone 2008, Shapcott 1997). As a result, detailed population genetic studies have not been possible. This study reports the development of EST microsatellite markers for this ancient and relictual podocarp species, *Lagarostrobos franklinii*. These markers will be useful for studying existing patterns of genetic diversity and how they relate to climatic fluctuations during the glacial-interglacial cycles and past human impacts and also will also provide insight into the importance of asexual reproduction.

Materials and Methods

RNA-seq data of *L. franklinii* was obtained from the Plant One-KP Project repository (http://www.onekp.com). This data consisted of 22,888,026 paired end reads of 90 bp in length. *De novo* assembly was undertaken in CLC Genomics Workbench 8.5.1 (Qiagen, Denmark) and the 52,802 resultant contigs (N50 = 1344) were mined for microsatellite regions. Primers were then developed for each region using default settings in PrimerPro (http://webdocs.cs.ualberta.ca/~yifeng/primerpro/). Microsatellites were targeted if they consisted of over 8 tandem repeats and the microsatellite was located >25 bp from the beginning or end of the contig. These criteria resulted in 70 microsatellite primer pairs and each primer pair was tested for amplification in four samples. For all loci, the forward primer was synthesized with one of three different M13 sequences (5'-GCCTCCCTCGCGCCA-3', 5'-GCCTTGCCAGCCCGC-3', and 5'-CAGGACCAGGCTACCGTG-3'), and the reverse was tagged with a PIG-tail (5'-GTTTCTT-3'; (Brownstein 1996). PCR amplification was performed using the QIAGEN Multiplex PCR Kit (Qiagen, Germany) and consisted of a 10 µL reaction volume, containing approximately 5 ng of DNA, 5 µL of 2× Multiplex PCR Master Mix, 0.06 µM of forward primer, 0.1 µM of reverse primer, and 0.08 µM of fluorescently labeled M13 primer. The PCR profile consisted of an initial denaturation at 95 °C for 3 minutes; followed by 35 cycles of 95 °C for 30 seconds, 60 °C for 3 minutes, 68 °C for 1 minute; with a final 20-minute extension at 68 °C. The amplification products were separated by capillary electrophoresis on an ABI 3130 Genetic Analyzer (Life Technologies, Waltham, Massachusetts, USA) with the GeneScan 600 LIZ Size Standard (Life Technologies). Genotype calling was completed with GeneMarker software (SoftGenetics, State College, Pennsylvania, USA). Genetic analyses were undertaken in GenAlEx 6.5 (Peakall and Smouse 2006) and GENEPOP 4.2 (Raymond 1995).

A total of 50 primer pairs successfully amplified and were subsequently tested for scoring reliability and presence of polymorphism in eight samples representing the entire species range (data not shown). Ten of these loci were reliably scorable and displayed polymorphism. These loci were subsequently screened in 84 samples from three different populations encompassing the geographic range of *L. franklinii*: Corinna, on the Pieman River in Western Tasmania, the Denison River from the Franklin-Gordon Wild Rivers National Park and Lake Judd from the Southwest National Park (see Appendix 1 for further details). Additionally, 24 individuals of the sister species *Manoao colensoi* were collected to examine marker transferability. These were from the Okuru River on the South Island of New Zealand (n = 22) and the Dunedin Botanic Gardens (n = 2) (Table 1).

Table 1
Details of the *Lagarostrobos franklinii* and *Manoao colensoi* samples used for assessing the variability of 10 ESTs microsatellites. Accession numbers of pre-existing herbarium specimen's representative of each population sampled are provided.

Species	Location	n	GPS coordinates	Accession number
L. franklinii	Corinna	27	41°39'12.97"S, 145° 5'25.81"E	HO586492
L. franklinii	Denison River	30	42°36'40.24"S, 145°59'29.49"E	CBG 8901682.1
L. franklinii	Lake Judd	27	42°58'14.88"S, 146°25'47.38"E	CBG 8700443.1
M. colensoi	Okuru River	22	43°53'42.28"S, 168°55'52.96"E	OTA 71838-71847
M. colensoi	Dunedin Botanic Garden	1	45°51'39.77"S, 170°31'33.79"E	DBG 19930739 (B)
M. colensoi	Dunedin Botanic Garden	1	45°51'39.77"S, 170°31'33.79"E	DBG 20130104 (A)

Note: n = number of individuals sampled. Details of each *L. franklinii* specimen are available online at the Australasian Virtual Herbarium (http://avh.chah.org.au). Of the 22 *M. colensoi* samples collected at Okuru River, 10 were submitted and received accession numbers from the OTA. CBG = Australian National Herbarium, Canberra, Australian Capital Territory, Australia; HO = Tasmanian Herbarium, Hobart, Tasmania, Australia. DBG = Dunedin Botanic Gardens living collection, Dunedin, New Zealand; OTA = The Otago Regional Herbarium, Dunedin, New Zealand.

Lagarostrobos franklinii is known to propagate clonally (Gibson, Davies and Brown 1991, Pedley 1980) so identifying clonal individuals an important application of the 10 loci. Therefore, the multi locus probability of identity (PID) for the 10 loci, that is, the probability that when two individuals drawn at random from a population will have the same genotype (Waits, et al. 2001), was calculated in Gimlet version 1.3.3 (Valiere 2002). Three PID estimates outlined by Waits et al. (2001) were estimated: biased PID which assumes individuals mate randomly; unbiased PID which corrects for sampling a small number of individuals and, sibs PID which assumes the population is composed of siblings, a possible scenario in *L. franklinii* due to inbreeding in some stands (Shapcott 1997).

Results and Discussion

In total, 10 loci could be reliably scored and displayed polymorphism (Table 2). The ten loci had between two and thirteen alleles in all 84 samples, with five loci having more than four alleles. The average number of alleles ranged from 3.0 to 4.7 per population, and the average observed heterozygosity and expected heterozygosity across the three populations was 0.39 (0.34-0.42) and 0.51 (0.37-0.44), respectively (Table 3). No significant deviations from Hardy–Weinberg equilibrium expectations were detected for any loci except for Huon_24367 in the Corinna population ($P = 0.0044$). Additionally, allele frequencies appeared independent among loci except for Huon_23863 with Huon_50967 and Huon_2030 in the Corinna population ($P < 0.0001$).

Multi-locus probability of identity values were below the threshold value (0.01) considered by Waits et al. (2001) to reliably distinguish between individual genotypes even under the sibs PID (Table 4). This indicates that our markers will be effective for identifying both sexually derived individuals in populations where inbreeding is prevalent and individuals derived from clonal reproduction.

None of the *M. colensoi* samples produced amplified products that could be reliably scored using the 10 loci, despite multiple attempts. Additionally, testing the DNA using universal primers (Internal Transcribed Spacer (ITS)) produced clear PCR products indicating that DNA quality was not causing the failures. The failure of markers to transfer is probably due to the deep evolutionary divergence between *M. colensoi* and *L. franklinii* estimated to have occurred approximately 64 mya (Biffin 2011).

We have developed ten polymorphic microsatellite markers, the first reported, for *L. franklinii*. The markers will be useful for examining both range-wide level processes shaping the species genetic diversity and population level processes including the role of asexual reproduction in the regeneration of *L. franklinii*.

Table 2
Characteristics of the 10 microsatellite markers developed for *Lagarostrobos franklinii.*

Locus		Forward Primer (5'-3')	Repeat Motif	Allele Size Range (bp)	BLASTX Top Hit Description	Genbank Accession	E value
Huon_23318	F:	GCGAGGTTCAAGGTGATGAT	(TA)8	217-253	-	MK423885	-
	R:	TGAGGACTTTCGGTTGCTCT					
Huon_50967	F:	AATTCGCTTTGAGCCAAGAA	(TC)18	164-196	-	MK423890	-
	R:	AGAGGCTTGCCTTGTCAAAA					
Huon_2030	F:	ATGTGCTTGTGAACCCCTGT	(AG)9	394-408	unknown [Picea sitchensis] ABR17991.1	MK423881	0
	R:	TTGAGGCAATCCTTTGGAAC					
Huon_23863	F:	CCAGTTCAGTCAGAAGCCG	(AT)10	273-295	-	MK423886	-
	R:	TTCCATTCCAGCATTGTTGA					
Huon_18442	F:	TAACTATGGGCTCCTCCACG	(CT)9	144-170	unknown [Picea sitchensis] ABR16594.1	MK423884	3.00E-07
	R:	AACCATGCAAAGAGGAATGG					
Huon_44177	F:	GGACCACATATTTGCAGAAACA	(AC)6(AT)6	255-257	-	MK423889	-
	R:	GAAGAATTGGAAATATGGCACA					
Huon_3945	F:	GGTTTGCAGGATCTATCAAAAA	(AT)9	303-341	unknown [Picea sitchensis] ABK23978.1	MK423882	1.00E-04
	R:	AGAAGCTTCGGTGAGAACCA					
Huon_13112	F:	TCCTGTTTAGCTCCAAATGCT	(AT)12	255-265	-	MK423883	-
	R:	GCCTACCGAGTCCATGAAAA					
Huon_24367	F:	GGAAGTGCATGGGAAGGTAA	(AT)13	291-297	-	MK423887	-
	R:	ATGGCCCTAACACTCTGGTG					
Huon_29065	F:	AAACTAGGACACCCATCCCC	(TA)8	127-135	-	MK423888	-
	R:	GATTCCCCCTCTCTCACACA					

- No significant similarity found

Table 3
Genetic diversity of the 10 polymorphic nuclear microsatellites assessed across three populations of *Lagarostrobos franklinii.*

	Corrina (n=27)			Denison Rv (n=30)			Lake Judd (n=27)			All Samples (n=84)		
Locus	A	Ho	He	A	Ho	He	A	Ho	He	A	Ho	He
Huon_23318	5	0.30	0.29	8	0.37	0.40	4	0.56	0.74	13	0.40	0.58
Huon_50967	4	0.37	0.37	5	0.57	0.61	2	0.04	0.04	7	0.33	0.40
Huon_2030	3	0.56	0.59	3	0.63	0.62	3	0.15	0.14	3	0.45	0.60
Huon_23863	3	0.52	0.50	3	0.33	0.28	2	0.04	0.04	4	0.30	0.44
Huon_18442	6	0.78	0.77	6	0.33	0.30	3	0.33	0.47	8	0.48	0.61
Huon_44177	1	0.00	0.00	2	0.07	0.06	1	0.00	0.00	2	0.02	0.02
Huon_3945	3	0.59	0.52	8	0.70	0.62	4	0.74	0.60	8	0.68	0.62
Huon_13112	5	0.78	0.73	5	0.60	0.66	4	0.62	0.64	6	0.65	0.76
Huon_24367	3	0.29	0.58	4	0.37	0.54	3	0.52	0.53	4	0.37	0.63
Huon_29065	1	0.00	0.00	3	0.20	0.24	4	0.41	0.51	4	0.20	0.40
Average	3.40	0.42	0.44	4.70	0.42	0.43	3.00	0.34	0.37	5.90	0.39	0.51

Note: A = number of alleles; He = expected heterozygosity; Ho = observed heterozygosity; n = number of individuals sampled. Locality and voucher information is provided in Appendix 1.

Table 4
The probability of identity (PID) values for each of the 10 polymorphic loci.

	PID by locus			Cumulative PID		
Locus	biased	unbiased	sibs	biased	unbiased	sibs
Huon_23318	0.201	0.189	0.512	0.2009	0.1887	0.5122
Huon_50967	0.377	0.364	0.645	0.07572	0.06866	0.3305
Huon_2030	0.225	0.218	0.504	0.01703	0.01495	0.1666
Huon_23863	0.382	0.374	0.624	0.0065	0.005588	0.104
Huon_18442	0.178	0.167	0.488	0.001156	0.0009329	0.05074
Huon_44177	0.954	0.952	0.977	0.001103	0.0008885	0.04955
Huon_3945	0.214	0.208	0.491	0.0002362	0.0001846	0.02434
Huon_13112	0.095	0.089	0.395	0.00002254	0.00001643	0.009619
Huon_24367	0.217	0.212	0.491	0.000004899	0.000003475	0.004723
Huon_29065	0.424	0.416	0.657	0.000002078	0.000001445	0.003103

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